Remarks

At the outset, Applicants invite the Examiner's attention to co-pending U.S. Patent Application Serial No. 11/100817.

The claims have been amended to conform to elected species. Claims 3, 23, 45 have been withdrawn because these claims are drawn to non-elected species, and their dependent claims amended accordingly. Other claims have been amended for reasons of clarity. Support for the amendments can be found throughout the specification as originally filed. No new matter is introduced by these amendments. Applicants reserve the right to prosecute any withdrawn subject matter at a later date or in a timely filed divisional application.

Claim Objections

On page 2 of the Office Action mailed February 22, 2007, the Examiner objected to claims 1-3, 5-23, 25-42, 44-45, 47-53 and 55 "as drawn to non-elected species." The claims have been amended to conform to the species election of "hematopoietic stem cells." Hence, this objection may be withdrawn.

Rejection under 35 U.S.C. § 103(a)

The Examiner, on page 4 of the Office Action rejects claims 1-42, 59-66, and 70-76 "as being unpatentable over U.S. Patent No. 5,741,899 ... Capon et al., in view of Spencer et al. (1996) Current Biology, Vol. 6(7), 839-847 and Blau et al. (1996) Blood, vol. 88 (10 Suppl. 1 part 1-2), p542A, meeting abstract (Blau 1996)." Applicants traverse the rejection.

The claim invention provides for a method for rendering a subpopulation of mammalian hematopoietic stem cells susceptible to drug-induced growth, proliferation or differentiation, by transducing hematopoietic stem cells with a recombinant DNA construct that encodes a fusion protein that includes at least one signaling domain and at least one drug-binding domain which is heterologous with respect to the signaling domain and binds to a selected drug, then exposing the transduced cells to a concentration of the drug effective to induce association of two or more fusion proteins, thereby inducing growth, proliferation or differentiation of the cells, wherein the transduction is carried out either *in vivo* or after the cells have been removed from the mammal from which the cells originated, and wherein the transduced cell is suitable for introduction into a mammal.

The claimed invention provides for pharmacologically-responsive fusion proteins that are useful for specifically and reversibly expanding genetically modified hematopoietic stem cell populations. This allows, for the first time, application of a neutral, targeted approach that does not rely on either negative selective pressure (e.g., antibiotic resistance) or addition of a growth factor that might have systemic implications, to provide selective positive inducement for cell growth, proliferation and/or differentiation.

Regarding Capon, on page 5 of the Office Action, the Examiner acknowledges Capon "does not provide specific guidance for the concentration of inducer to administer in order to achieve dimerization of the chimeric proteins resulting in cell proliferation." But Capon is deficient for several reasons and could not supply one of skill in the art with any reason to follow its teachings. As Applicants have pointed out before, if one skilled in the art simply followed the teachings of Capon, they would discover that the assays taught therein did not work. Confronted with this failure, the skilled artisan would not know why the assays failed. There could be many reasons for failure, given that Capon did not provide any examples other than prophetic examples to demonstrate the functionality of the described chimeric proteins. Hence, one following the teachings of Capon would have to experiment with various parameters of the assay to discover why it did not work. For example, there could be a problem with the dimerizing agent itself. If the dimerizing drug does not bring the chimeric proteins sufficiently close to one another, or alternatively, brings them too close to one another, signal transduction would not occur. Additionally, fusing a signaling domain to a heterologous inducing domain might, upon dimerization, orient the signaling domain in a way that prevents it from assuming the conformation necessary to transmit a signal. Because Capon did not demonstrate the actual functionality of any of the described constructs, it would be reasonable for one skilled in the art, faced with a failed assay, to question whether the constructs were capable of functioning at all.

Indeed, Capon suggests JAK2 dimerization as a means for transmitting a growth signal. However, others skilled in the art, *see, e.g.*, Mohi et al., 9 Mol. Biol. Cell 3299-308 (1998) (submitted herewith), found that JAK2 dimerization could not induce a growth signal unless the majority of the molecule (containing domains JH2 -JH7) was deleted from the signaling protein. The inventors had the same experience (unpublished). In contrast, the JH1 domain of JAK2 is capable of transmitting dimerization dependent growth signal in Ba/F3 cells. *Id.*; Zhao et al., 10(3) Molecular Therapy 456-68 (2004). Similarly, Capon refers to the use of other members of the JAK kinase family, however the inventors have tested all of the members of this family (JAK1,

JAK2, JAK3, TYK2) and found that only the JH1 domain of JAK2 was able to transmit a proliferative signal in primary hematopoietic cells (unpublished). In sum, although the Examiner cites Capon's guidance regarding use of saturating concentration of FK1012 as its sole deficiency, there is far more lacking in Capon than guidance for the concentration of inducer.

Spencer does not make up for the deficiencies of Capon. The Examiner asserts, on page 6, that "while signaling through the Fas receptor induces cell death rather than proliferation, the essential teaching of Spencer is that FK1012 can be effectively used as a synthetic inducer of dimerization of chimeric receptor proteins comprising FKBP domains, that such dimerization leads to functional signaling through the receptor, and that the determination of concentrations of FK1012 capable of inducing dimerization was routine." The Examiner provides not one shred of evidence that one of ordinary skill would equate apoptosis with the claimed outcomes: growth, proliferation, and/or differentiation.

Moreover, Spencer refers to mouse thymocytes, and does not even mention the application of the techniques to hemopoietic stem cells. Nor does Spencer suggest that transduced cells are suitable for introduction into a mammal. Furthermore, Spencer refers only to the use of FK1012, which would ***not*** be appropriate for treating cells transduced *in vivo*, or as a CID for cells suitable for introduction suitable for cells (i.e., for *in vivo* therapy), because FK1012 may bind to endogenous FKBP12 sequences. Such binding is undesirable for several reasons. For example, endogenous FKBP12 may act as a sink, sequestering the drug so that it cannot perform its task of binding FKBP12 motifs in the fusion protein. Importantly, binding to endogenous protein might interfere with the physiologic involvement of FKBP12 in processes such as calcium-channel function and sperm motility. FKBP12 knockout mice were found to have severe congenital cardiomyopathy, indicating that FKBP12 has a role in the development and perhaps the maintenance of the myocardium. *See* Neff & Blau, 97(9) Blood, 2535-40, (2001). Hence, one skilled in the art would not read Spencer to suggest FKBP12, which may prove toxic *in vivo*, as a model for dosing.

In contrast, the present specification does provide numerous CID alternatives to FK1012, thus preventing binding to endogenous FKBP12, throughout the document. *See, e.g.*, in particular, Example 10 regarding AP1903.

Thus, Spencer does not support the Examiner's contention, on page 6, that "at the time of filing, the optimization of drug concentrations for dimerization of chimeric proteins was routine and well-developed," in the context of the claimed invention, which refers to, *inter alia*,

hematopoietic stem cells susceptible to drug-induced growth, proliferation or differentiation, the transduction of mammalian hematopoietic stem cells; wherein the transduction is carried out either *in vivo*, or after the cells have been removed from the mammal such that the transduced cells are suitable for introduction into a mammal.

Regarding Blau (1996), this Abstract refers to *in vitro* studies using the murine IL3-dependant Ba/F3 cell line. Although an *in vitro* system may serve as a model for study or discovery, it does not follow that every model can serve as a basis for extrapolation to a desired downstream *in vivo* application, let alone embody the teaching to achieve such an application. Indeed, it was well known in the art at the time of filing of the application that established (immortalized) cell lines are limited as models for the behavior of primary cells (Gene VI, Benjamin Lewin, Oxford Universal Press In., New York 1997); and, by extension, even more so for primary cells destined for introduction into a mammal. Blau further reports that "after IL-3 was withdrawn, each clone exhibited a dose-dependent proliferative response to FK1012." Clearly such selective pressure is ***not*** reproducible *in vivo*, and evidences another deficiency with this reference. As noted in previous responses to the Examiner, there are many examples (including those cited in Blau, in which signaling molecule capable of transmitting a proliferative signal in Ba/F3 cells ***fail*** to function in primary cells. Hence, one of ordinary skill could not extrapolate from Blau, even in combination with Capon and Spencer, to achieve the claimed methodology.

In contrast, the instant specification provides the necessary additional teaching, for example, *see* Example 8, wherein primary marrow cells were transduced *in vitro* with a construct encoding fusion protein and then introduced into mice. The mice were then treated with drug which binds to the fusion protein encoded by the construct in the transplanted primary cells and cell expansion of specific cell types was demonstrated. The ability to apply the teachings of the application to human hematopoietic cells was also demonstrated (see Examples 9 and 10) and there is also provided a tissue culture model system distinct to that disclosed in Blau as "a more direct way to study signaling pathways ..." (see pages 53-55 of the specification).

Hence, Applicants respectfully suggests that these references are not sufficient to support an obviousness rejection, and request that this rejection be withdrawn.

The Examiner, on page 7 of the Office Action, rejects claims 44-53 and 55-58 under 35 U.S.C. § 103 "as being unpatentable over ... Capon et al., in view of U.S. Patent No. 5,994,313 (11/30/99), hereafter referred to as Crabtree et al., Spencer ... and Blau (1996)." Applicants

traverse the rejection. Basically, the Examiner asserts that Crabtree teaches various *in vitro* assays varying the concentration of FK1012 to determine effective concentrations for oligomerizing chimeric receptors comprising FKBP12 and a signaling domain, and further teaches methods to optimize dosages of the inducer drug for *in vivo* administration.

Capon, Spencer and Blau, singly and combined, are deficient for the reasons expressed above. Crabtree does not make up for these deficiencies. Crabtree relates to apoptosis, with the goal of "regulatably initiating cell death in engineered cells." Col. 2, lines 58-64. The cells of Crabtree are transduced primarily so that at the desired time the cells can be destroyed by introduction of the CID. "Modified cells of this invention, which are capable of expressing a primary chimeric protein containing a domain such as the cytoplasmic domain of the Fas antigen or TNF receptor ... are readily eliminated through apoptosis following exposure of the cells to a ligand capable of oligomerizing the primary chimeras." There is no discussion of any motivation to provide for cell growth, proliferation, or differentiation outside of that context. Furthermore, Crabtree does not provide for the application of its approach to hemopoietic stem cells, using only immortalized Jurkat cell line. As noted above, although an immortalized cell line is often a valuable model, it does not always correlate with stem cells. Hence, there is nothing in Crabtree that suggests how one would modify the techniques to provide for the instant invention.

The claimed invention is the culmination of numerous inventive steps, none of which are described in any of the articles cited by the examiner. Initially, the inventors used Ba/F3 cells as a screening method to identify molecules capable of transmitting a proliferative signal. As described in the Blau 1996 Abstract, the first molecule examined was the erythropoietin receptor. After describing this finding in the Blau 1997 PNAS paper, the inventors searched for other receptors that were candidates for demonstrating activity in hematopoietic stem cells, and tested c-kit (Jin et al., 91(3) Blood 890-97 (1998)), Flt-3 (Jin et al., 95(14) P.N.A.S. 8093-97 (1998)), and Mpl (Jin et al., 1998). Of these four initial candidates, Mpl demonstrated the activity, as described in the instant specification. The erythropoietin receptor construct functioned best in the context of three FKBP12 domains, and induced only a minimal growth promoting effect when tested in the context of a single FKBP12 motif (Blau et al., 1997). At the time, the inventors accurately predicted that the use of a single drug binding domain would be desirable because multiple repeated sequences might promote recombination in the context of retroviral vectors, and that retroviral vectors (a class which includes lentivirus vectors and human foamy virus vectors) were useful for the transduction of primary hematopoietic cells.

Additional technical hurdles posed by retroviral transduction were exemplified by the inventors studies using c-kit, which promoted FK1012-dependent proliferation in Ba/F3 cells following stable transfection (Jin et al., 1998), but which could not transmit a growth signal when transferred in the context of a retroviral vector, a problem very likely due to the propensity of retroviral vectors to recombine. Flt3, as well as many other receptors that were subsequently tested, induced a strong proliferative signal in Ba/F3 cells, but exerted minimal activity in primary hematopoietic cells (Jin et al., 1998, Zeng et al., 98(2) Blood 328-34 (2001)). Thus the examples provided in the instant specification were not "obvious" but were hard-won achievements realized following extensive experimentation.

Indeed, combining the cited references fails to provide a "combinati[on of] previously known elements." *KSR Int'l Co v. Teleflex Inc.*, No. 04-1350 (April 30, 2007) at 14, 15. In summary, comparing all of the cited references, in combination, to the claimed invention, it is clear that claimed invention reflects an advancement and "real innovation." *KSR Int'l*, at 15. Applicants respectfully request that the § 103 rejection be withdrawn.

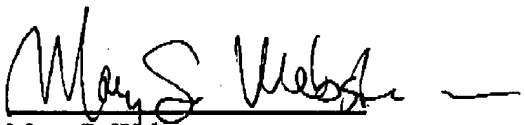
Conclusion

Applicants respectfully request reconsideration of this application and allowance of the pending claims in view of the above remarks.

Except for issue fees payable under 37 C.F.R. §1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. §§1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account No. 50-0310. This paragraph is intended to be a **CONSTRUCTIVE PETITION FOR EXTENSION OF TIME** in accordance with 37 C.F.R. §1.136(a)(3).

Date: August 21, 2007

Respectfully submitted,



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Molecular Biology of the Cell
Vol. 9, 3299–3308, December 1998

Activation and Functional Analysis of Janus Kinase 2 in BA/F3 Cells Using the Coumermycin/Gyrase B System

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Submitted June 8, 1998; Accepted September 15, 1998
Monitoring Editor: Keith Yamamoto

Janus kinase 2 (Jak2) protein tyrosine kinase plays an important role in interleukin-3– or granulocyte–macrophage colony-stimulating factor–mediated signal transduction pathways leading to cell proliferation, activation of early response genes, and inhibition of apoptosis. However, it is unclear whether Jak2 can activate these signaling pathways directly without the involvement of cytokine receptor phosphorylation. To investigate the specific role of Jak2 in the regulation of signal transduction pathways, we generated gyrase B (GyrB)–Jak2 fusion proteins, dimerized through the addition of coumermycin. Coumermycin induced autophosphorylation of GyrB–Jak2 fusion proteins, thus bypassing receptor activation. Using different types of chimeric Jak2 molecules, we observed that although the kinase domain of Jak2 is sufficient for autophosphorylation, the N-terminal regions are essential for the phosphorylation of Stat5 and for the induction of short-term cell proliferation. Moreover, coumermycin-induced activation of Jak2 can also lead to increased levels of c-myc and CIS mRNAs in BA/F3 cells stably expressing the Jak2 fusion protein with the intact N-terminal region. Conversely, activation of the chimeric Jak2 induced neither phosphorylation of Shc or SHP-2 nor activation of the c-fos promoter. Here, we showed that the GyrB–Jak2 system can serve as an excellent model to dissect signals of receptor-dependent and -independent events. We also obtained evidence indicating a role for the N-terminal region of Jak2 in downstream signaling events.

INTRODUCTION

The Janus kinase (Jak) family of cytoplasmic protein tyrosine kinases, composed of Jak1, Jak2, Jak3, and Tyk2, has a kinase domain in the C terminus (JH1), a pseudokinase domain (JH2), and a large N-terminal region with highly conserved Jak homology domains (JH3–JH7). The N-terminal region of the Jaks appears to be responsible for interactions with the β subunit of hematopoietic growth factor receptors, whereas the kinase domain is required for functional activity (Frank *et al.*, 1995; Zhao *et al.*, 1995; Chen *et al.*, 1997). By bringing at least two Jak molecules in close proximity, Jaks become activated likely through transphosphorylation. Like other tyrosine kinases, phosphorylation of tyrosine residues within the activation loop of the kinase domain is probably an essential part of this activation (Feng *et al.*, 1998). The activated Jaks subsequently phosphorylate tyrosine residues of cytokine receptors as well as a variety of substrates, most notably the Stat family of transcription factors (Ihle, 1996). Upon phosphorylation, Stats undergo dimerization and translocate to the nucleus where they bind to specific DNA sequence and enhance the transcription of specific genes (Darnell *et al.*, 1994).

Granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5 exert biological activities, such as cell proliferation, activation of early response genes, and inhibition of apoptosis, through heterodimeric receptors composed of α and β subunits (Arai *et al.*, 1990). The α subunit is specific for

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each receptor (Gearing *et al.*, 1989), whereas the β subunit (βc) is shared by all (Kitamura *et al.*, 1991). We reported that IL-3 and GM-CSF stimulate multiple signal transduction pathways through distinct cytoplasmic domains of the βc (Sakamaki *et al.*, 1992; Watanabe *et al.*, 1993b). The membrane proximal region, which contains the box1 motif, is involved in the induction of c-myc as well as in cell proliferation. In addition to the membrane proximal region, tyrosine residues in the membrane distal region are required for activation of the MAP kinase cascade leading to c-fos promoter activation (Itoh *et al.*, 1996, 1998). Experiments using dominant negative Jak2 revealed that all known activities of GM-CSF depend on the activation of Jak2 (Watanabe *et al.*, 1996), and the binding of overexpressed Jak2 to the membrane proximal region of βc-containing box1 was noted in insect cells (Quelle *et al.*, 1994). Because the activation of various signaling events by βc mutants indicated that the box1 region is essential for the activation of all the tested GM-CSF–induced phenomena (Itoh *et al.*, 1996), GM-CSF may exert its activities through Jak2 activation, which binds to the box1 region of βc. Downstream of Jak2, there are probably at least two different types of signaling pathways, one depending on the tyrosine phosphorylation of βc and the other arising from Jak2, without involvement of a receptor domain. In this study we focused on the signals derived from Jak2.

To isolate the activation of this protein tyrosine kinase aside from other growth factor-induced signals, we introduced the coumermycin/gyrase B (GyrB) system (Farrar *et al.*, 1996). Coumermycin acts as a natural dimerizer of GyrB, because it binds GyrB with a stoichiometry of 1:2. To generate a Jak2 molecule that could be induced to dimerize by coumermycin, we constructed chimeras involving GyrB and the entire Jak2 or GyrB fused to the Jak2 kinase region only. We report here that GyrB–Jak2 becomes activated in the presence of coumermycin, and the importance of the N-terminal region of Jak2 for downstream signaling events became evident.

MATERIALS AND METHODS

Reagents and Antibodies

The GyrB cDNA (pKS-GyrB) was kindly provided by Dr. R. Perlmutter (University of Washington, Seattle, WA). Cytokine-inducible SH2-containing protein (CIS) cDNA used as a probe in the Northern blot analysis was a gift from Dr. A. Yoshimura (Kurume University, Kurume, Japan). Coumermycin was obtained from Sigma Chemical (St. Louis, MO). G418 was a gift from Schering-Plough (Kenilworth, NJ). The anti-Jak2 antibody (C-20), mainly used for immunoprecipitation, anti-Stat1, anti-Stat3, anti-Stat4, anti-Stat5a, anti-Stat5b, anti-Stat6, and anti-Src homology 2-containing protein-tyrosine phosphatase (SHP-2) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-phosphotyrosine antibody (4G10), anti-Shc antibody, and anti-Jak2 antibody, used for Western blotting, were purchased from Upstate Biotechnology (Lake Placid, NY). Fetal calf serum (FCS) was purchased from Biocell Laboratories (Carson, CA). RPMI 1640 medium was purchased from Nikken BioMedical Laboratories (Kyoto, Japan). Recombinant murine IL-3 (mIL-3) expressed in silkworm, *Bombyx mori*, was purified as described elsewhere (Miyajima *et al.*, 1987).

Construction of Plasmids

Jak2 cDNA was originally cloned into the pME18S vector, which has the SRα promoter (Takebe *et al.*, 1988), as described (Watanabe *et al.*, 1996). There are seven conserved regions in Jak family proteins, which are referred to as JH1–JH7 starting with the most C-terminal end (Ihle and Kerr, 1995). JH1 and JH2 correspond to kinase and pseudokinase domains, respectively. The cDNA for GyrB fused to the 5′ end of N terminus–truncated Jak2 containing JH2 and JH1 regions (GNJK) was constructed by replacing the JH7 to JH3 region (*Bstx*I–*Msc*I fragment blunt ended using T4 DNA polymerase) of pME18S-Jak2 with GyrB sequence (*Not*I–*Spe*I fragment blunt ended using the Klenow fragment) of pKS-GyrB. The cDNA for GyrB inserted between the JH3 and JH2 domains of full-length Jak2 (GIJW) was prepared by inserting GyrB (blunt-ended *Sac*I-*Spe*I fragment from pKS-GyrB) into the *Msc*I site of pME18S-Jak2. To construct the cDNA for GyrB fused to the N-terminus of whole Jak2 (GNJW), the coding region of GyrB was isolated from pKS-GyrB at *Not*I and *Eco*RV sites and blunt ended using the Klenow fragment. The fragment was then ligated into *Bstx*I-digested pME18S-Jak2, which was also blunt ended using T4 DNA polymerase. All the constructs were verified by restriction enzyme digestion and by dideoxy sequencing using an automated sequencer (Applied Biosystems, Foster City, CA).

Cell Culture and Transfections

COS7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FCS, 50 units/ml penicillin, and 50 μg/ml streptomycin. Transient transfection of plasmids into COS7 cells was done by using the DEAE-dextran method (Maniatis *et al.*, 1982). A mIL-3–dependent proB cell line, BA/F3 (Palacios and Steinmetz, 1985), was maintained in RPMI 1640 medium supplemented with 5% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.25 ng/ml mIL-3. To obtain stable transfectants, BA/F3 cells were cotransfected with 13.5 μg of GNJK, GIJW, or GNJW plasmids together with 1.5 μg of pKU-2Neo vector, containing the neomycin resistance gene, by electroporation, as described (Watanabe *et al.*, 1995a). After selection in 1 mg/ml G418 for ~15 d, drug-resistant clones were screened for protein expression by Western blotting using anti-Jak2 antibody.

Immunoprecipitation and Western Blotting

Immunoprecipitation and Western blotting were done as described (Itoh *et al.*, 1996). Briefly, cells (1×10^7 cells per sample) were harvested and lysed in 500 μl of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 100 μM Na_3VO_4, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin A) for 1 h at 4°C. Cell lysates were incubated for 2 h at 4°C together with the indicated antibody and protein A-Sepharose beads (Pharmacia, Piscataway, NJ). The immunoprecipitates were separated on a 7% SDS-polyacrylamide gel and transferred electrophoretically to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% bovine serum albumin (Fraction V, Sigma), the membrane was subjected to Western blot analysis with appropriate antibodies and visualized using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, United Kingdom) as described previously (Watanabe *et al.*, 1996).

Luciferase Assay

BA/F3 cells stably expressing various GyrB–Jak2 fusion proteins (1×10^6 cells per sample) were transfected with c-fos–luciferase

DNA (Watanabe *et al.*, 1993a) (1 μg per sample) or β-casein–luciferase (Wakao *et al.*, 1994) (3 μg per sample) by electroporation, as described (Watanabe *et al.*, 1995a). The cells were maintained in mIL-3 medium for ~12 h, and then mIL-3 was depleted for 6 h, followed by stimulation with mIL-3 (1 ng/ml), coumermycin (1 μM), or novobiocin (1 μM) for 6 h, and then harvested. Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL), and the luciferase activity was measured using a luminometer (model LB9501; Berthold Lumat, Tokyo, Japan) and a luciferase assay substrate (Promega, Madison, WI). All values are expressed relative to the protein concentration.

Cell Proliferation Assay

Cell proliferation was measured by [^{3}H]thymidine incorporation, as described (Watanabe *et al.*, 1993a). Briefly, cells were seeded into 96-well plates (1×10^4 cells per well) with various concentrations of coumermycin or mIL-3. After 24 h of culture, [^{3}H]thymidine (1 μCi/well) was added, followed by incubation for another 3 h before harvest. Cells were harvested onto a glass fiber filter, and [^{3}H]thymidine incorporation was measured using a filter counter (model 1450 MicroBeta; Wallac, Turku, Finland).

DNA Fragmentation Assay

To detect DNA fragmentation, 5×10^6 cells were cultured for 16 h in 5% FCS-containing RPMI 1640 medium, the same medium supplemented with 1 ng/ml mIL-3, or 1 μM coumermycin. Low-molecular-weight chromosomal DNA was isolated using the ApopLader Ex kit (Takara Biomedicals, Shiga, Japan) according to the manufacturer's instructions and electrophoresed through a 2% agarose gel. DNA fragments were visualized by ethidium bromide staining.

Northern Blot Analysis

Northern blots were performed with mRNA prepared using the Fast Track 2.0 kit (Invitrogen, San Diego, CA). Briefly, 1 μg of mRNA was separated on a 1% agarose gel containing 6% formaldehyde and transferred onto a nylon membrane (Hybond-N, Amersham) by capillary blotting. The blots were hybridized with cDNA probes (c-myc, CIS, and glyceraldehyde-3-phosphate dehydrogenase genes) labeled by the Ready-To-Go kit (Pharmacia) using [α-^{32}P]dCTP. The blotted membrane was visualized using a Fuji BAS-3000 image analyzer (Tokyo, Japan).

RESULTS

Construction and Expression of GyrB–Jak2 Fusion Proteins

To examine the specific role of Jak2 in signal transduction, we used a coumermycin-induced chemical dimerization method (Farrar *et al.*, 1996). We constructed three different fusion genes in which GyrB was fused to Jak2. Figure 1A shows a schematic diagram of fusion proteins GNJK (GyrB fused to the 5′ end of the JH2–JH1 fragment of Jak2), GIJW (GyrB is inserted between JH2 and JH3 of full-length Jak2), and GNJW (GyrB fused to the 5′ end of full-length Jak2). The size and level of fusion protein expression were examined in COS7 cells by immunoprecipitation followed by Western blotting using anti-Jak2 antibody. GNJK was detected at ~100 kDa, and GIJW and GNJW migrated at ~160 kDa, being consistent with the predicted molecular mass (Figure 1B). The constructs were then used to generate stable transfectants in BA/F3 cells. The sizes and levels of the transfected fusion proteins in BA/F3 stable cell lines were confirmed by immunoprecipitation followed by Western blotting, using a Jak2-specific antibody (Figure 2A, bottom panel).

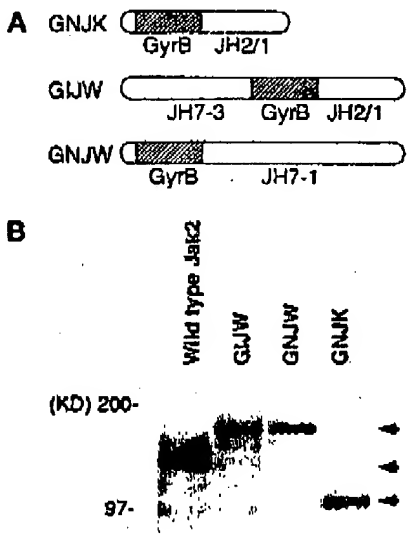

Figure 1. Expression of GyrB–Jak2 fusion proteins in COS7 cells. (A) Schematic diagram of the GyrB–Jak2 fusion protein constructs. Open bar, region from Jak2; hatched bar, GyrB sequence. In GNJK, GyrB is fused to the 5′ end of the JH2–JH1 region of Jak2. In GIJW and GNJW, GyrB is inserted between the JH3 and JH2 regions or fused at the N terminus of full length Jak2, respectively. (B) Expression of GNJK, GIJW, GNJW and the wild-type Jak2 (as control) were examined in COS7 cells. Immunoprecipitation followed by Western blotting was done using anti-Jak2 antibody. The positions of the expressed Jak2 and GyrB–Jak2 fusion proteins and molecular mass standards are shown.

Coumermycin-induced Tyrosine Phosphorylation of GyrB–Jak2 Fusion Proteins in BA/F3 Cells

Because tyrosine phosphorylation of Jak2 closely correlates with the tyrosine kinase activity of Jak2 (Witthuhn *et al.*, 1993; Ihle, 1995; Nakamura *et al.*, 1996), we first examined the tyrosine phosphorylation status of the GyrB–Jak2 fusion proteins by immunoprecipitation followed by Western blotting with anti-phosphotyrosine antibody (4G10). As shown in Figure 2A, addition of coumermycin induced tyrosine phosphorylation of the GNJK (100 kDa) and the GIJW (160 kDa) fusion proteins but not the GNJW fusion protein or the endogenous Jak2. The tyrosine-phosphorylated bands close to the position of endogenous Jak2 appearing in the 6th and 10th lanes were different from endogenous Jak2, because these bands migrated slightly slower than the endogenous Jak2. The monomeric coumarin

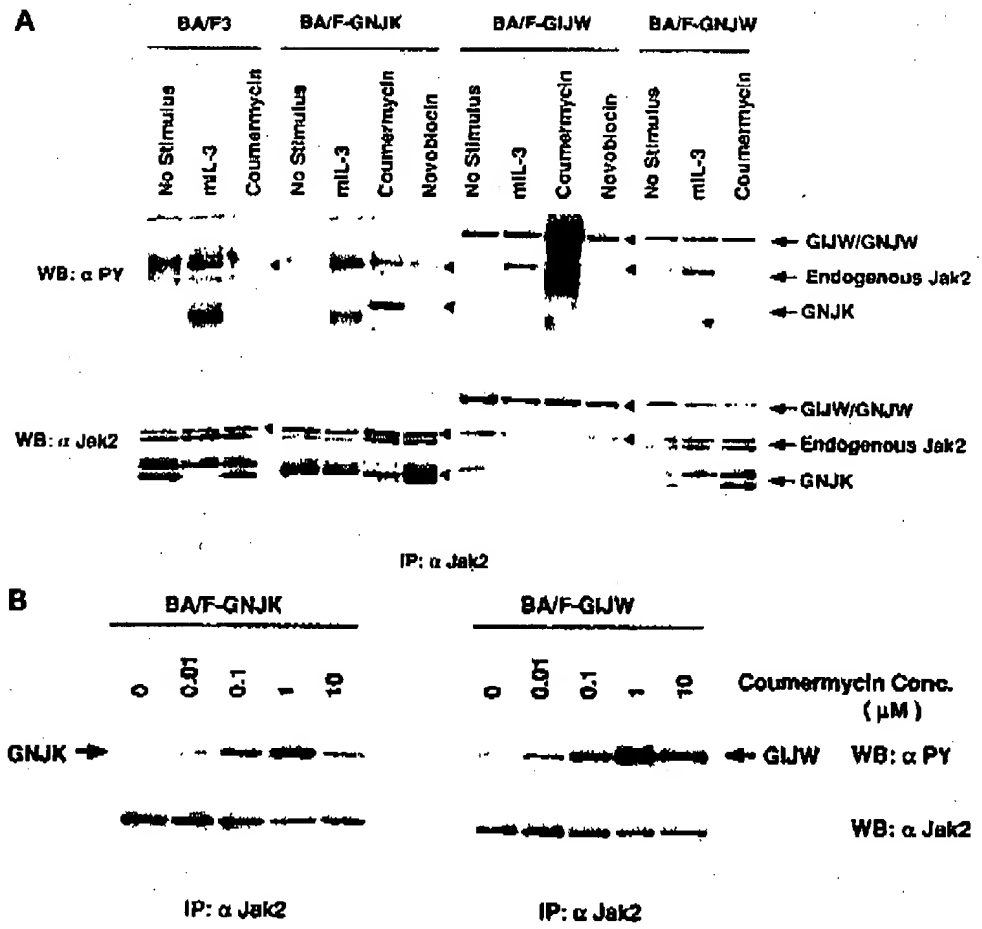

Figure 2. Coumermycin-induced tyrosine phosphorylation of GyrB–Jak2 fusion proteins in BA/F3 transfectants. (A) Tyrosine phosphorylation of GyrB–Jak2 in BA/F-GNJK, -GIJW, or -GNJW cells. The factor-depleted cells were stimulated with 1 ng/ml mIL-3, 1 μM coumermycin, or 1 μM novobiocin for 15 min. Jak2 immunoprecipitants were blotted with either anti-phosphotyrosine antibody (top panel, α PY) or anti-Jak2 antibody (bottom panel). The arrows indicate the 160-kDa GIJW/GNJW bands, the 130-kDa endogenous Jak2 band, and the 100-kDa GNJK band. (B) Dose response of coumermycin-induced tyrosine phosphorylation of GyrB–Jak2 proteins. The factor-depleted BA/F-GNJK and BA/F-GIJW cells were stimulated for 15 min with the indicated amounts of coumermycin. (C) Tyrosine phosphorylation of mIL-3 receptor β subunit. Immunoprecipitation was done using anti-mIL-3 receptor β subunit, and the membrane was blotted with either anti-phosphotyrosine antibody (top panel, α PY), or anti-β antibody (bottom panel).

antibiotic novobiocin did not induce GNJK and GIJW phosphorylation, as expected. The endogenous Jak2, but not the fusion proteins, was tyrosine phosphorylated after mIL-3 stimulation. In BA/F-GIJW and -GNJW cells, fusion proteins but not endogenous Jak2 were slightly tyrosine phosphorylated, even in the absence of mIL-3 or coumermycin, possibly because of overexpression of the fusion proteins. The tyrosine phosphorylation status of the GNJW fusion protein was not changed after coumermycin stimulation, possibly because the GyrB fused at the N terminus of the entire Jak2 molecule could not bring the kinase domains of two Jak2 molecules close enough to cross-phosphorylate each other after induction with coumermycin. Because GNJW is not phosphorylated in response to coumermycin, BA/F-GNJK and BA/F-GIJW cells were used for the following experiments.

We next examined the coumermycin dose dependence of GyrB–Jak2 phosphorylation. The maximum activation occurred at a concentration of 1 μM coumermycin (Figure 2B) in both BA/F-GNJK and BA/F-GIJW cells. The level of phosphorylation induced by 10 μM coumermycin was less than that induced by 1 μM coumermycin. These results are

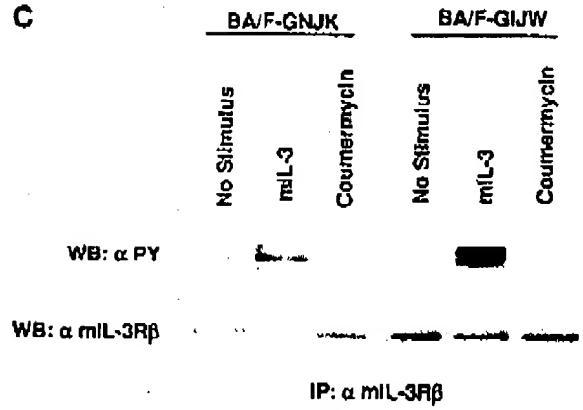

Figure 2 (cont).

consistent with the idea that excess amounts of coumermycin may prevent dimer formation because of 1:1 rather than 1:2 stoichiometrical binding of coumermycin and fusion proteins. Taken together, these results suggest that coumermycin induces phosphorylation of the GyrB–Jak2 protein by dimerization. To analyze whether GyrB–Jak2 phosphorylates the endogenous mIL-3 receptor, we checked tyrosine phosphorylation of the mIL-3 receptor β subunit by immunoprecipitation followed by Western blotting using anti-phosphotyrosine antibody. As shown in Figure 2C, the mIL-3 receptor β subunit is tyrosine phosphorylated in response to stimulation with mIL-3 in both cells but is not phosphorylated by the addition of coumermycin. This result indicates that the coumermycin-induced activation of the GyrB–Jak2 fusion protein bypasses the receptor activation.

Stat5 but neither SHP-2 nor Shc Was Tyrosine Phosphorylated in BA/F-GIJW Cells

Because Jak2 is responsible for the phosphorylation of a variety of cellular proteins after IL-3 and GM-CSF stimulation (Watanabe *et al.*, 1995b), we next attempted to identify molecules that were tyrosine phosphorylated after coumermycin-induced activation of GyrB–Jak2 fusion proteins. We first examined Stat5, which is phosphorylated after Jak2 activation by mIL-3 stimulation in BA/F3 cells (Mui *et al.*, 1995). The tyrosine phosphorylation of Stat5 was investigated by immunoprecipitation, using anti-Stat5a and anti-Stat5b antibodies, followed by Western blotting with anti-phosphotyrosine antibody (Figure 3A). The phosphorylation was observed only in BA/F-GIJW cells, not in BA/F-GNJK cells. Therefore, the JH3–JH7 regions of Jak2, in addition to the kinase and pseudokinase domains, are involved in Stat5 phosphorylation. We also examined whether other members of Stats could be activated in these cells by immunoprecipitation using specific Stat antibodies followed by Western blotting with anti-phosphotyrosine antibody. We did not observe any phosphorylation of Stat1, Sta 3, Stat4, and Stat6 after stimulation with mIL-3 or coumermycin in both BA/F-GNJK and BA/F-GIJW cells.

We next addressed the question of tyrosine phosphorylation of SHP-2 and Shc, because these have been implicated in the activation of the Ras–MAP kinase pathway leading to activation of the c-fos serum response element site (Watanabe *et al.*, 1997). Stimulation by mIL-3 resulted in tyrosine phosphorylation of SHP-2 and Shc (Figure 3B), as expected. In contrast, coumermycin stimulation did not induce tyrosine phosphorylation of SHP-2 or Shc in either BA/F-GNJK cells or BA/F-GIJW cells. Reprobing the Shc immunoprecipitant membrane with an anti-Grb2 antibody revealed that the adapter protein Grb2, which forms a complex with Shc after mIL-3 stimulation (Itoh *et al.*, 1998), could not be coimmunoprecipitated by coumermycin stimulation.

We further analyzed the functional activities of the Stat and MAP kinase cascades using a transient transfection analysis of β-casein and c-fos promoter fused to the luciferase gene. As shown in Figure 3C, β-casein–luciferase activity is induced in BA/F-GIJW but not in BA/F-GNJK cells in response to the addition of coumermycin. It is notable that the level of induction is far less than that observed with mIL-3 stimulation. Transient transfection assays using c-fos–luciferase as the reporter gene revealed that the c-fos promoter was not activated by stimulation with coumermycin in BA/F-GNJK or BA/F-GIJW cells (Figure 3D). Phosphorylated tyrosine residues of the cytoplasmic distal region of βc are essential for the recruitment and activation of both SHP-2 and Shc (Itoh *et al.*, 1996, 1998). Taken together, these results indicate that the activation of GyrB–Jak2 fusion protein is insufficient to activate the Ras–MAP kinase pathway leading to c-fos promoter activation.

Coumermycin-induced Growth of BA/F-GIJW Cells

We reported that Jak2 plays an essential role in cell proliferation (Watanabe *et al.*, 1996). To determine whether GyrB–Jak2 fusion proteins have growth-promoting function, we analyzed [^{3}H]thymidine incorporation in response to coumermycin in BA/F-GNJK and -GIJW cells. Cells were washed with depletion media and cultured in various concentrations of coumermycin or mIL-3 for 24 h. In response to coumermycin stimulation, incorporation of [^{3}H]thymidine was observed in BA/F-GIJW cells but not in BA/F-GNJK cells (our unpublished results). We next examined the coumermycin dose dependency for [^{3}H]thymidine incorporation in BA/F-GIJW cells. As

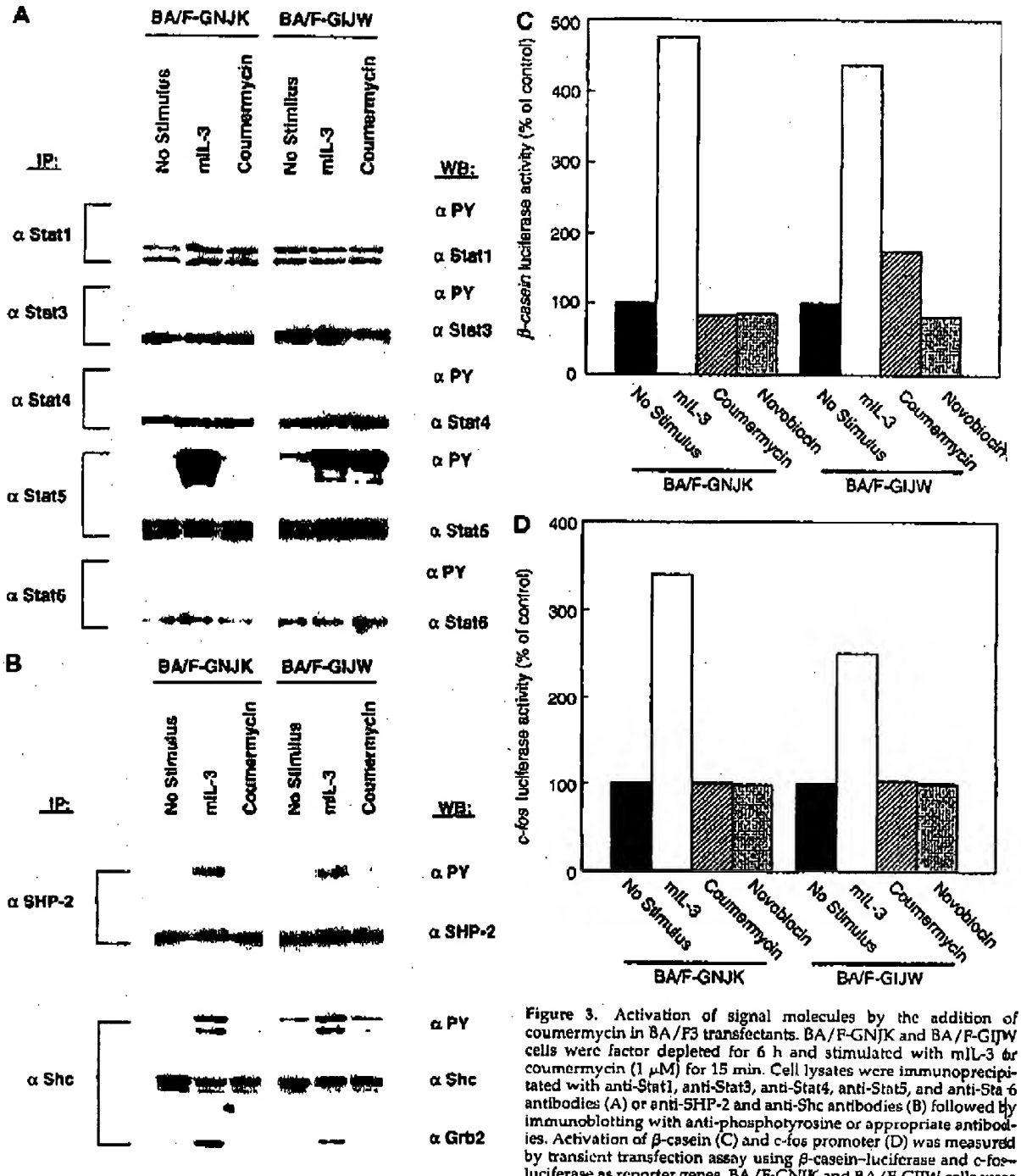

Figure 3. Activation of signal molecules by the addition of coumermycin in BA/F3 transfectants. BA/F-GNJK and BA/F-GIJW cells were factor depleted for 6 h and stimulated with mIL-3 or coumermycin (1 μM) for 15 min. Cell lysates were immunoprecipitated with anti-Stat1, anti-Stat3, anti-Stat4, anti-Stat5, and anti-Stat6 antibodies (A) or anti-SHP-2 and anti-Shc antibodies (B) followed by immunoblotting with anti-phosphotyrosine or appropriate antibodies. Activation of β-casein (C) and c-fos promoter (D) was measured by transient transfection assay using β-casein–luciferase and c-fos–luciferase as reporter genes. BA/F-GNJK and BA/F-GIJW cells were transfected with either β-casein–luciferase or c-fos–luciferase, and luciferase activities induced by 1 ng/ml mIL-3 (□), 1 μM coumermycin (▨), or 1 μM novobiocin (■) were analyzed as described in MATERIALS AND METHODS. The results are represented as a relative value of that of the unstimulated controls (■). Experiments were done at least three times, and essentially the same results were obtained.

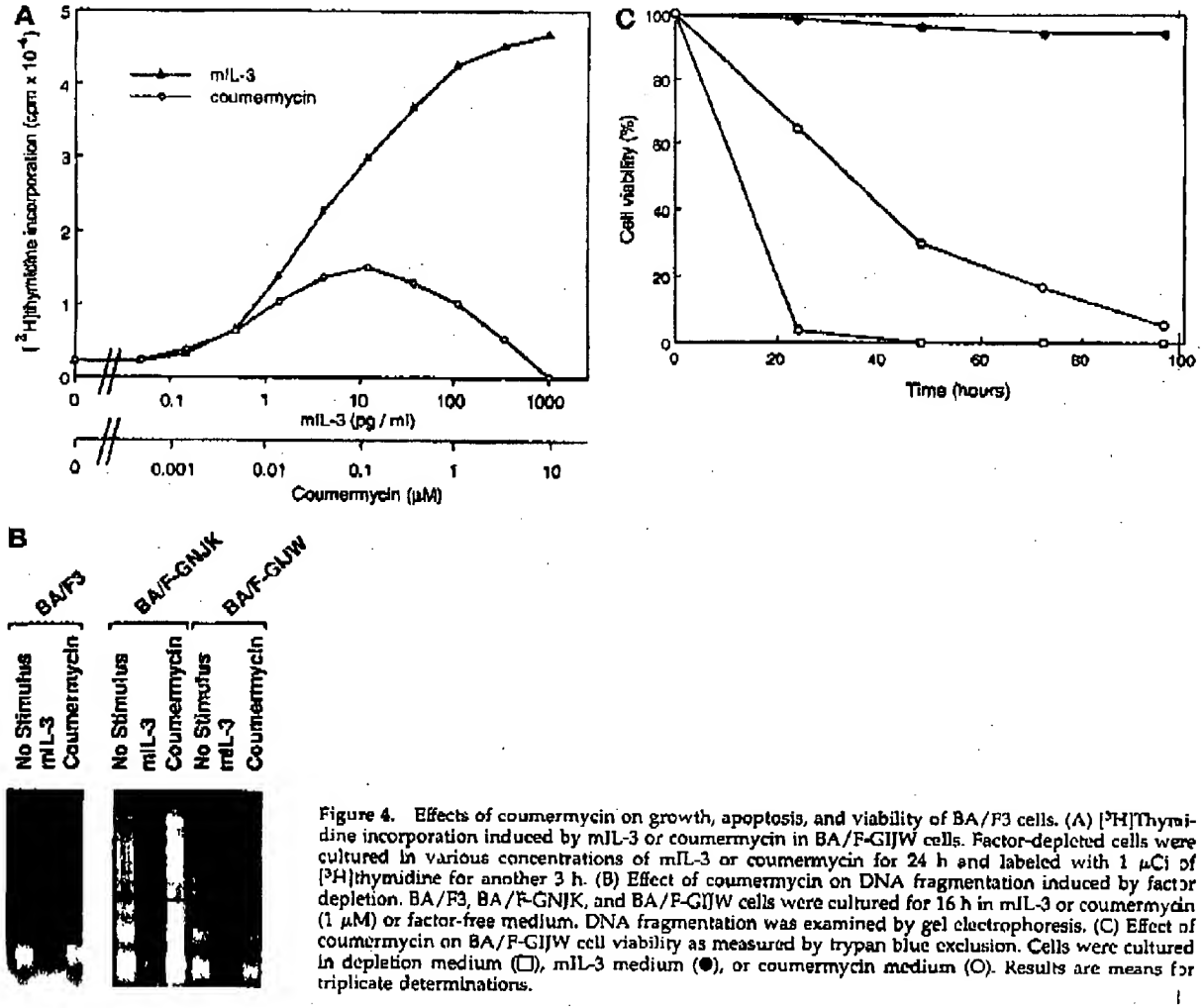

Figure 4. Effects of coumermycin on growth, apoptosis, and viability of BA/F3 cells. (A) [^{3}H]Thymidine incorporation induced by mIL-3 or coumermycin in BA/F-GIJW cells. Factor-depleted cells were cultured in various concentrations of mIL-3 or coumermycin for 24 h and labeled with 1 μCi of [^{3}H]thymidine for another 3 h. (B) Effect of coumermycin on DNA fragmentation induced by factor depletion. BA/F3, BA/F-GNJK, and BA/F-GIJW cells were cultured for 16 h in mIL-3 or coumermycin (1 μM) or factor-free medium. DNA fragmentation was examined by gel electrophoresis. (C) Effect of coumermycin on BA/F-GIJW cell viability as measured by trypan blue exclusion. Cells were cultured in depletion medium (□), mIL-3 medium (●), or coumermycin medium (○). Results are means for triplicate determinations.

shown in Figure 4A, coumermycin-induced [^{3}H]thymidine incorporation was observed even with 1.4 nM coumermycin and reached the maximum level at 0.12 μM coumermycin. The level of incorporation was lower than that observed in the case of mIL-3 stimulation, even with the optimal concentration of coumermycin. However, with higher doses of coumermycin there was a decline in activity, and the value returned close to the basal level with 10 μM coumermycin. These results further confirm that activation of GyrB–Jak2 proceeds through dimerization and that Jak2 activation can lead to proliferative signals.

We next tested the potential of GyrB–Jak2 fusion proteins to prevent apoptosis, measured by a chromosomal DNA fragmentation assay. Within 16 h of mIL-3 depletion, characteristic DNA fragmentation was observed in parental BA/F3 cells (Liu, Itoh, Arai, and Watanabe, unpublished data), and substantial DNA fragmentation was observed in both BA/F-GNJK and BA/F-GIJW cells by 16 h of factor depletion, as expected (Figure 4B). DNA fragmentation was prevented when the cells were cultured in the presence of mIL-3. In the presence of coumermycin, DNA fragmentation occurred even in BA/F-GIJW cells, which can stimulate [^{3}H]thymidine incorporation. Therefore, the activation of GyrB–Jak2 fusion protein is insufficient to prevent factor depletion-induced apoptosis.

We next tested the long-term cell viability of the BA/F-GIJW cell in the presence of coumermycin by trypan blue exclusion. Although virtually no cell was viable after 48 h of factor depletion, the presence of coumermycin kept a small but significant population of cells viable for >72 h (Figure 4C). After 100 h of

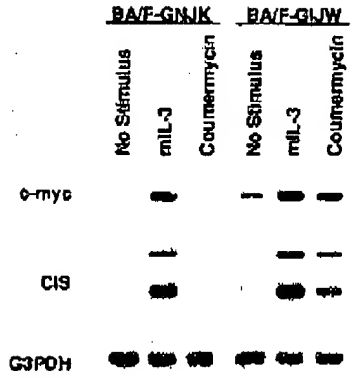

Figure 5. Coumermycin-dependent induction of c-myc and CIS mRNAs. BA/F-GNJK and BA/F-GIJW cells were factor depleted for 5 h and stimulated with 1 ng/ml mIL-3 or 1 μM coumermycin for 30 min. mRNA was extracted, and Northern blot analysis was performed using c-myc, CIS, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as probes.

culture, no cell was viable, indicating that GIJW can sustain short-term but not long-term proliferation or cell survival.

Induction of c-myc and CIS mRNA by Coumermycin in BA/F-GIJW Cells

c-myc is induced by mIL-3 and human GM-CSF (hGM-CSF) stimulation in BA/F3 cells expressing the hGM-CSF receptor (Watanabe *et al.*, 1993a). We reported previously that the membrane proximal region, including box1 and box2 of βc, is essential and sufficient to induce c-myc mRNA in BA/F3 cells (Watanabe *et al.*, 1995a, 1993b). This region is also essential for GM-CSF–induced phosphorylation of Jak2, and dominant negative Jak2 suppressed c-myc induction by hGM-CSF (Watanabe *et al.*, 1996). We next asked whether the activation of GyrB–Jak2 fusion proteins would lead to the induction of c-myc as determined by Northern blot analysis. As shown in Figure 5, coumermycin induced an increase in the level of c-myc mRNA expression in BA/F-GIJW but not BA/F-GNJK cells. The level of induction was slightly lower than that observed in the case of mIL-3 stimulation. CIS, a negative regulator of cytokine signals, is another immediate-early gene that may be a target of the Jak–Stat pathway (Yoshimura *et al.*, 1995). Because the coumermycin-induced activation of GyrB–GIJW proved to be sufficient to activate Stat5, we next examined the induction of CIS in these cells by Northern blot analysis. The addition of mIL-3 resulted in the activation of the CIS gene in both BA/F-GNJK and BA/F-GIJW cells. Induction of CIS mRNA was observed in BA/F-GIJW but not in BA/F-GNJK cells by coumermycin stimulation.

DISCUSSION

In the present study, we analyzed the mechanisms of Jak2 activation and its specific role in signal transduction pathways. For this we made use of GyrB–Jak2 chimeric proteins, which can be artificially dimerized by antibiotics without involving ligand–receptor systems. Because ligand binding to cytokine receptors apparently induces dimerization of the receptor subunits, leading to transactivation of associated Jak molecules (O'Shea, 1997), we speculated that Jak2 could possibly be activated by artificial forced dimerization. Such an approach has been successfully used to activate Raf-1 kinase, using a GyrB–Raf1 fusion protein (Farrar *et al.*, 1996). We found that coumermycin induced phosphorylation of GyrB–Jak2 without the induction of endogenous Jak2 phosphorylation. Therefore, it is evident that dimerization or aggregation of Jak2 is important for its activation. Although GyrB–Jak2 was activated, the βc was not phosphorylated upon coumermycin stimulation. So, we speculate that the activation of Jak2 may not be sufficient to phosphorylate the βc chains. Also, SHP-2 and Shc were not tyrosine phosphorylated by coumermycin, possibly because the receptor βc was not phosphorylated. These observations support the findings that phosphorylated tyrosine residues of the cytoplasmic distal region of the βc are essential for phosphorylation of both SHP-2 and Shc (Itoh *et al.*, 1996, 1998). Thus, we isolated signaling downstream of Jak2 from growth factor receptor–dependent signals.

We observed that GyrB–Jak2 specifically activated Stat5. No other members of Stats could be phosphorylated upon coumermycin stimulation, indicating that the fusion protein has some specificity in phosphorylating the cellular substrates. It is believed that activation of Stat by cytokine stimulation requires tyrosine residues of the cytokine receptor (Stahl *et al.*, 1995; Ihle, 1996). Our findings imply that Stat5 can be activated without the involvement of receptor tyrosine residues. To support this view, it was reported that there may be two distinct pathways for Stat5 activation, one by creating tyrosine-phosphorylated docking sites at the cytokine receptor and the other by direct interaction between Jaks and Stat5 (Fujitani *et al.*, 1997). We found the induction of Stat5 phosphorylation was diminished but not completely abrogated when all eight tyrosines of the βc were mutated (Itoh *et al.*, 1998). It therefore seems feasible that in growth factor signaling, two distinct pathways act synergistically for the maximum activation of Stat5.

We reported the essential role of Jak2 in GM-CSF–dependent cell proliferation using dominant negative Jak2 (Watanabe *et al.*, 1996). In this study, coumermycin-induced activation of GyrB–Jak2 can lead to cell proliferation in BA/F-GIJW cells. However, the level of incorporation of [^{3}H]thymidine by coumermycin

was lower than that observed in the case of mIL-3 stimulation, and proliferation was only short term. Using a series of βc mutants containing mutated tyrosine residues, we showed the requirement of receptor tyrosine residues to promote proliferation. Any single tyrosine residue maintains the ability to induce proliferation (Itoh *et al.*, 1998); thus lack of cell proliferation in the present system may be explained by lack of receptor involvement. Factor depletion–induced apoptosis occurred even in the presence of coumermycin implied that GyrB–Jak2 activation is insufficient to prevent apoptosis. In an apparent contradiction with our present finding, a previous report (Sakai and Kraft, 1997) demonstrated that activation of CD16–Jak2-kinase domain fusion is sufficient to prevent cells from apoptosis and showed constitutive phosphorylation of Shc, which also has been implicated in transducing signals to the Ras–MAP kinase pathway. Our series of analysis of antiapoptosis activity of hGM-CSF using various βc mutants revealed that Jak2 activation through the box 1 region is essential, and that activation of either a tyrosine kinase inhibitor genistein-sensitive pathway or the MAP kinase cascade is sufficient to sustain cell viability (Liu, Itoh, and Watanabe unpublished data). Thus, the ability of the CD16–Jak2 chimera to prevent cells from apoptosis may be facilitated through the MAP kinase pathway which in downstream of Shc activation. The most prominent difference between GyrB–Jak2 and CD16–Jak2 may be in the subcellular localization of the fusion proteins. It is possible that although Jak2 alone is insufficient, with the help of other signaling molecules presumably located at the membrane, it can induce antiapoptotic signals. This hypothesis may also explain the lack of SHP-2 and Shc phosphorylation.

Our results show that c-myc and CIS are targets of Jak2 activation, because the presence of coumermycin led to an increase in the levels of c-myc and CIS mRNAs. However, it remains to be determined how the activation of Jak2 ultimately leads to the activation of c-myc. It has been reported that putative E2F binding sites at the P2 region of the c-myc promoter play an important role in mIL-3– or hGM-CSF–induced activation of c-myc (Watanabe *et al.*, 1995a). It is therefore tempting to speculate that Jak2 activates the c-myc promoter by altering the composition of E2F complexes. But how Jak2 alters the composition of E2F complexes is not clear.

We also found that the N-terminal domains of Jak2 are required for the activation of Stat5 as well as for induction of c-myc and CIS genes, because they are induced only in BA/F-GIJW cells. Therefore, the N-terminal regions (JH3–JH7) of Jak2 thought to be important for binding of the cytokine receptor are also required for activation of some signaling events possibly by binding or interacting with other signaling molecules required for induction of such events. Future efforts toward defining the functional aspects of all the JH regions may elucidate mechanisms of signal transduction mediated by Jak2.

ACKNOWLEDGMENTS

We thank T. Itoh, A. Muto, M. Dahl, Y. Sakurai, and R. Liu for helpful discussion, Y. Aoki for excellent technical assistance, and M. Ohara for helpful comments.

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